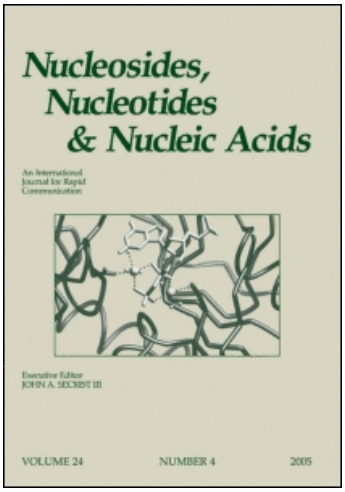

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Synthetic Nucleosides and Nucleotides. 40. Selective Inhibition of Eukaryotic DNA Polymerase α by 9-(β-D-Arabinofuranosyl)-2-(*p*-*n*-butylanilino)adenine 5'-Triphosphate (BuAaraATP) and Its 2'-Up Azido Analog: Synthesis and Enzymatic Evaluations

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SYNTHETIC NUCLEOSIDES AND NUCLEOTIDES. 40. SELECTIVE INHIBITION OF EUKARYOTIC DNA POLYMERASE α BY 9-(β-D-ARABINOFURANOSYL)-2-(*p-n*-BUTYLANILINO) ADENINE 5'-TRIPHOSPHATE (BuAaraATP) AND ITS 2'-UP AZIDO ANALOG: SYNTHESIS AND ENZYMATIC EVALUATIONS † [1]

Aki Tomikawa, Masaki Seno, Kunie Sato-Kiyotaki, Chizuru Ohtsuki, Toshiaki Hirai, Toyofumi Yamaguchi *, Takeo Kawaguchi [a], Shonen Yoshida [b] and Mineo Saneyoshi

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ABSTRACT: Starting from 2',3',5'-tri-*O*-acetyl-2-iodoadenosine, 9-(β-D-arabinofuranosyl)-2-(*p-n*-butylanilino)adenine and its 2'(*S*)-azido counterparts were synthesized in seven steps. These exhibited only moderate growth-inhibitory effects against mouse leukemic P388 cells (IC_{50} = 13-24 μM), although 5'-triphosphate derivatives showed strong and selective inhibitory action on calf thymus DNA polymerase α, but not on β- and ε-polymerases from eukaryotes.

Aphidicolin-sensitive DNA polymerases (pols), α, δ and ε, generally known as the pol α-family, play important roles in nuclear DNA replication and repair.[2] For investigating the properties and functions of these enzymes, various inhibitors have been synthesized and used for study.[3] It has been reported that 2-(*p-n*-butylanilino)-2'-deoxyadenosine 5'-triphosphate (BuAdATP, **9a**) and its guanine counterpart, 2-(*p-n*-butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP) (FIG. 1), were selective and potent inhibitors of eukaryotic pol α.[3] These two compounds inhibit pol α with *Ki* values in the nanomolar range by competing with the normal substrate dATP or dGTP, respectively. In contrast, pols δ and ε are not inhibited by these compounds.[4-6]

† This paper is dedicated to the memory of Professor Tsujiaki Hata.

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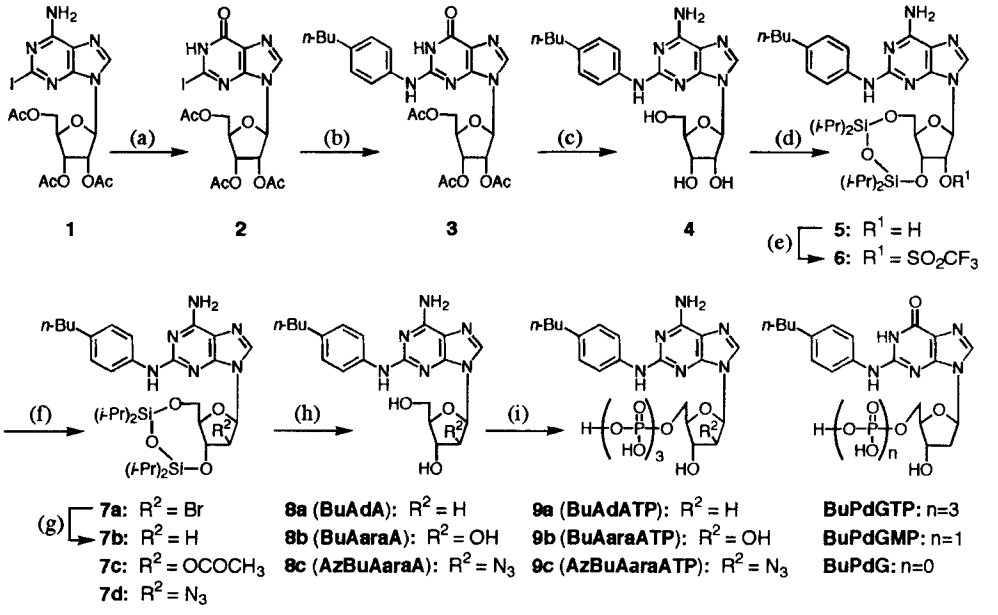

FIG. 1. Synthetic route of 2'-up substituted BuAdA analogs and their 5'-triphosphates, and the structures of the BuPdGs. (a) $NaNO_2$, aqueous CH_3COOH; (b) *p*-(*n*-butyl)aniline, CH_3OH; (c) 1) $POCl_3$, *N*,*N*-dimethylaniline, Et_4NCl, CH_3CN, 2) NH_3-CH_3OH; (d) $TIPDSCl_2$, imidazole, DMF; (e) CF_3SO_2Cl, DMAP, Et_3N, pyridine; (f) LiBr, NaOAc or LiN_3 in HMPA; (g) *n*-Bu_3SnH, AIBN, toluene; (h) *n*-Bu_4NF, THF; (i) 1) $POCl_3$, $(EtO)_3PO$, 2) 1,1'-carbonyldiimidazole, DMF, 3) tri-*n*-butylammonium pyrophosphate, DMF.

Therefore, BuAdATP and BuPdGTP have been widely used for discrimination and identification of enzymes in the pol α-family.

Since these polymerases play important roles in DNA replication, they are thought to be promising target enzymes in the development of new anticancer agents. However, it has also been reported that the nucleosides, BuAdA and BuPdG, did not exhibit significant cytotoxic activity *in vitro*.[7] It is well known that the 2'(*S*)-substituted nucleoside analogs, arabinofuranosylcytosine and 2'-azido-2'-deoxyarabinofuranosylcytosine, exhibit significant anticancer activity.[8-10] The glycosyl bonds of arabino-nucleosides have been shown to be more stable chemically and more resistant to enzymatic cleavage than the corresponding 2'-deoxynucleosides.[11] In addition, the affinities of arabino-nucleoside 5'-triphosphates for eukaryotic pols were

shown to be higher than those of natural dNTPs.[12,13] Therefore, synthesis and biological evaluation of the sugar-modified BuAdA analogs (**8b** and **8c**) and their 5'-triphosphates (**9b** and **9c**) have been considered.

Synthesis

For the synthesis of purine nucleosides bearing a *p-n*-butylanilino group at the C-2 position, triacetyl-2-iodoadenosine (**1**)[14], which could readily be prepared from guanosine in four synthetic steps, was considered to be suitable as a starting material. However, Marumoto *et al.* have reported that the reaction of 2-chloro or 2-bromoadenosine with anilines gave none of the desired product, or produced it in a very low yield.[15] Heating of 2-iodoadenosine with *p-n*-butylaniline in 2-methoxyethanol yielded a small amount of 2-(*p-n*-butylanilino)adenosine, however, the product could not be isolated. Marumoto *et al.* also reported that 2-bromoinosine is a useful starting material for introduction of an anilino group at the 2-position.[15] Similarly, Wright *et al.* introduced a butylanilino group to the 2-position of purine ring using 2-bromohypoxanthine.[4] Therefore, **1** was first converted to triacetyl-2-iodoinosine (**2**) by deamination reaction in 84% yield. Heating a mixture of **2** and *p-n*-butylaniline in methanol yielded triacetyl-2-(*p-n*-butylphenyl)guanosine (**3**) in 75% yield. Chlorination of **3** followed by treatment with methanolic ammonia gave 2-(*p-n*-butylanilino)adenosine (**4**). Modification of the sugar moiety at the 2'-position was performed by the method of Fukukawa *et al.*[16] After protection of the 3' and 5' hydroxyl groups of **4** with a tetraisopropyldisiloxyl group, the 2'-alcohol (**5**) was converted to its triflate **6**. Nucleophilic substitution of triflate **6** with LiBr, NaOAc and LiN_3 in HMPA gave the 2'(*S*)-substituted analogs **7a**, **7c** and **7d**, respectively. According to the standard method, reduction of **7a** with tri-*n*-butyltin hydride yielded the 2'-deoxy derivative **7b**. Removal of the silyl protecting groups of **7b**, **7c** and **7d**, afforded 2-(*p-n*-butylanilino)-2'-deoxyadenosine (**8a**, BuAdA)[7], the arabino analog (**8b**, BuAaraA) and the 2'-up azido analog (**8c**, AzBuAaraA), respectively. Compounds **8b** and **8c** were converted to the corresponding 5'-triphosphates **9b** (BuAaraATP) and **9c** (AzBuAaraATP), by selective phosphorylation with $POCl_3$[17] in triethylphosphate followed by the phosphoroimidazolidate method[18].

Biological and Biochemical Evaluations

We examined the antileukemic activity of nucleosides **8a**, **8b** and **8c** against murine leukemic P388 cells *in vitro*. The IC_{50} values of these compounds were 16.3 (**8a**), 24.2 (**8b**) and 13.6 μM (**8c**), respectively.

Next, we investigated the inhibitory effects of BuAaraATP (**9b**) and AzBuAaraATP (**9c**) on calf thymus pol α[19], cherry salmon testis pol β[20] and pol ε with activated DNA as a template/primer. As shown in Figure 2, both analogs **9b** and **9c** showed potent inhibitory activity on calf thymus pol α. The modes of inhibitory action by these compounds were competitive with respect to dATP (Km = 3.0), and the Ki values of **9b** and **9c** were 0.017 and 0.038 μM, respectively. In the case of cherry salmon pol α[21], the enzyme displayed a Ki of 0.007 μM for BuAaraATP and a Km of 2.5 μM for dATP (data not shown). The inhibitory effects of **9b** were comparable to that of BuAdATP (**9a**) (Ki = 0.0026 and 0.008 μM) reported by Khan *et al.*[6,22] These results suggest that pol α recognizes BuAaraATP as well as BuAdATP, but cannot discriminate between 2'-up H and OH of dATP analogs bearing a butylanilino group at the C-2. On the other hand, 70% of pol ε activity remained in the presence of 50 μM BuAaraATP (**9b**) or AzBuAaraATP (**9c**). Therefore, **9b** and **9c** were found to be weak inhibitors of pol ε, and moderate inhibitors of pol β. These results also agree with those for BuAdATP[6]. Thus, it is suggested that dATP analogs **9b** and **9c** inhibit pol α selectively, and that these may be employed for distinguishing pol α from other vertebrate pols as well as BuPdGTP and BuAdATP.

The results of the primer extension assays are shown in Figure 3. These experiments were performed using a 25- and 14-mer template/primer capable of incorporating dATP at the first, and dGTP at the second position from the 3'-end of the primer. The primer extension experiment in the presence of dATP gave a product that migrated as 15-mer (lane 3), and in the presence of both dATP and dGTP gave a 16-mer product (lane 4), as expected. When examined in the presence of BuAaraATP instead of dATP (lanes 5, 6 and 7), the original 14-mer mostly remained, and a faint band was detected at a position corresponding to approximately 16-mer. This band, which was afforded in 3.5-4.5% yield from Cerenkov counting analysis after excising the

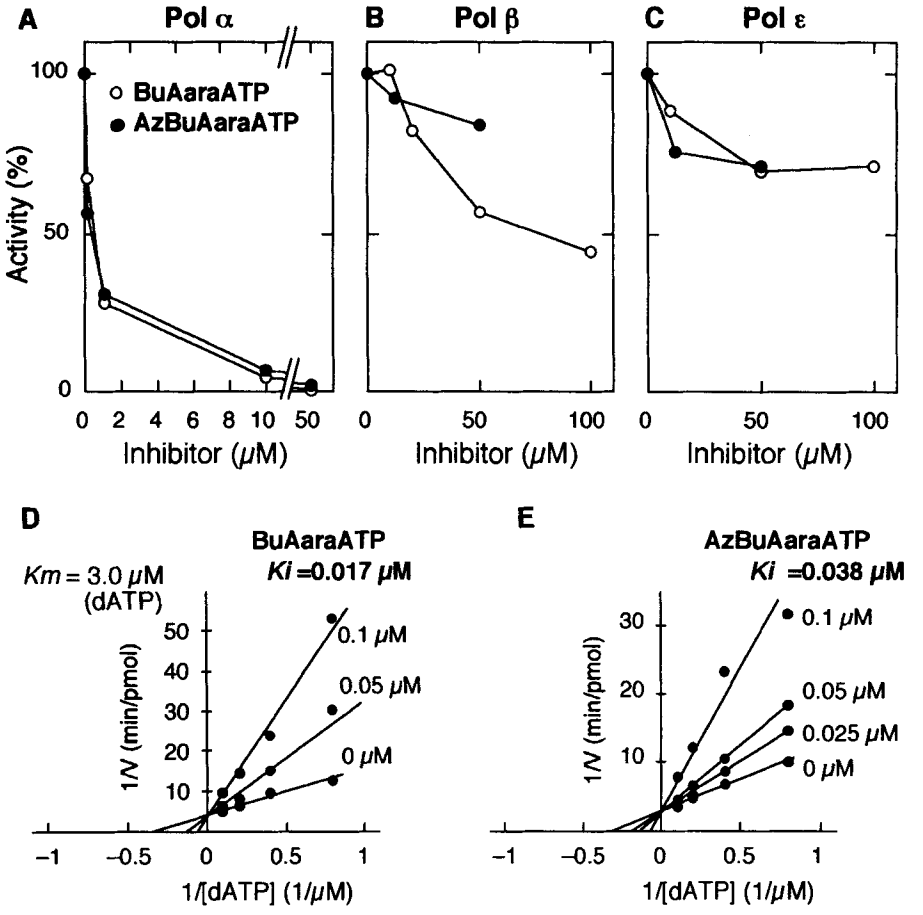

FIG. 2. Inhibitory effects of BuAaraATP (**9b**)(○) and AzBuAaraATP (**9c**)(●) on calf thymus DNA polymerase (pol) α (panel A), cherry salmon testis pols β (panel B) and ε (panel C), and Lineweaver-Burk plots for the inhibition of pol α by **9b** (panel D) and **9c** (panel E). Reactions were carried out for 20 min at 37°C with activated DNA as the template/primer in the presence of 50 μM [^{3}H]dATP (panels A, B and C) or various concentrations of [^{3}H]dATP (panels D and E).

radioactive band from the gel, was judged to be 15-mer bearing a BuAaraAMP residue at the 3'-terminus, since no bands were detected between the original 14-mer and the newly produced faint band. The slow migration of the band in the gel was consistent with the observation that migration of the oligomer bearing the butylphenyl group at N-2 of the dGMP residue was slower than that of the nonmodified oligomer[23]. When the next substrate dGTP was present in the reaction, similar results were also obtained (lanes 8, 9

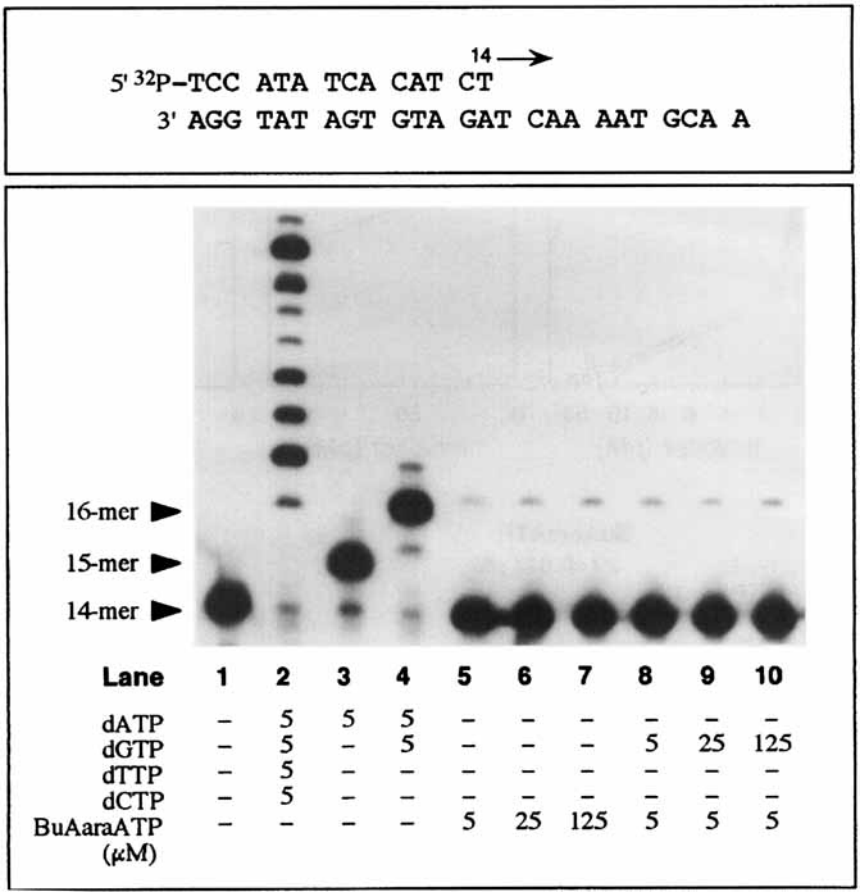

FIG. 3. Primer extension reactions with calf thymus DNA polymerase (pol) α in the presence of BuAaraATP (**9b**). *Upper panel*, structure of the template/primer. *Lower panel*, assays described in the Experimental Section utilized a 5' ^{32}P-labeled 14-mer/25-mer duplex as the template/primer with the indicated additions.

and 10). These findings suggest that BuAaraATP is a poor substrate for pol α, and that incorporation of BuAaraAMP at the 3'-terminus of the primer strand might cause chain termination. The former result differed from the observation that BuPdGTP was not incorporated into growing DNA by pol α.[22]

Unfortunately, nucleosides **8b** and **8c** did not significantly inhibit the growth of murine leukemic P388 cells in culture, although their 5'-triphosphate derivatives were shown in the present study to be potent and selective inhibitors of cellular pol α.

Presumably, these compounds would be poor substrates for cellular kinases. In order to circumvent the first activation step by cellular kinases, we are attempting to synthesize the 5'-monophosphate and phosphonate derivatives of the nucleosides described here, and evaluate their biological activities.

Experimental Section

^{1}H-NMR spectra were recorded on a JEOL FX90Q (90 MHz) spectrometer with $(CH_3)_4Si$ as an internal standard. Mass spectra were measured on a JMX-SX 102A spectrometer. Elemental analyses were performed on a Perkin Elmer Series II CHNS/O analyzer 2400. Melting points were determined on a Yanaco Model MP-J3 apparatus and are uncorrected. HPLC analysis was performed using a Shimadzu LC-9A apparatus fitted with a YMC pack ODS A-302 (YMC Co. Ltd, 4.6 mm x 15 cm) reverse-phase column, and the columns were run at 45 °C. Elution was with 30% (v/v) CH_3CN containing 0.05 M triethylammonium acetate (TEAA) (pH 7.0) at a flow rate of 1 ml/min, and detection was performed at 280 nm (Method A). [^{3}H]dATP, [^{3}H]dTTP and [γ-^{32}P]ATP were purchased from Amersham. The 14-mer and 29-mer oligodeoxyribonucleotides were purchased from Nihon Techno Service.

2',3',5'-tri-*O*-Acetyl-2-iodoinosine (2). To a solution of 2',3',5'-tri-*O*-acetyl-2-iodoadenosine (**1**)[14] (10 g, 19.3 mmol) in a mixture of acetic acid (200 ml) and water (20 ml) was added sodium nitrite (16 g, 230 mmol). Addition of sodium nitrite was repeated 8 times every 0.5 h in portions of 2.0 g with vigorous stirring at room temperature. The solution was then allowed to stand for 12 h at room temperature. After evaporation of the solvent, the residue was dissolved in a mixture of $CHCl_3$ (200 ml) and water (100 ml). The organic phase was separated, washed with saturated $NaHCO_3$, and then with water, and dried over $MgSO_4$. After evaporation of the solvent, the residue was crystallized from ethanol to give **2** as yellowish fibers (8.51 g, 84.9%): mp 167—168 °C. UV (CH_3OH) λ_{max} 272 and 252 nm. ^{1}H-NMR ($CDCl_3$) δ: 2.11, 2.14, 2.16 (s x 3, 9 H, acetyl x 3), 4.40 (m, 3 H, H-5' and 4'), 5.58 (dd, 1 H, H-3'), 5.76 (dd, 1 H, H-2'), 6.11 (d, 1 H, H-1'), 7.89 (s, 1 H, H-8). *Anal.* Calcd. for $C_{16}H_{17}IN_4O_8$: C, 36.94; H, 3.29; N, 10.77. Found: C, 37.15; H, 3.36; N, 10.49.

2',3',5'-Tri-*O*-acetyl-2-(*p*-*n*-butylphenyl)guanosine (3). A mixture of **2** (8.76 g, 15.9 mmol) and *p*-*n*-butylaniline (18.7 ml) in methanol (73 ml) was heated for 4 h at 95 °C. After the resulting solution had stood at room temperature overnight, the precipitates were collected by filtration and crystallized from CH_3OH afforded **3** as pale yellowish fibers (6.53 g, 75.9%): mp 233—235 °C. UV (CH_3OH) λ_{max} 280 nm. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (t, 3 H, CH_3 of butyl), 1.2-1.8 (m, 4 H, CH_2 x 2 of butyl) 2.01, 2.07, 2.16 (s x 3, 9 H, acetyl x 3), 2.56 (t, 2 H, Ar-CH_2), 4.0-4.4 (m, 3 H, H-5' and 4'), 5.38 (m, 1 H, H-3'), 5.95 (br s, 2 H, H-1' and 2'), 7.16 and 7.57 (d x 2, 2 H x 2, aromatic), 7.69 (s, 1 H, H-8), 9.45 (s, 1 H, NH). *Anal.* Calcd. for $C_{26}H_{31}N_5O_8$: C, 57.66; H, 5.77; N, 12.93. Found: C, 57.57; H, 5.70; N, 13.13.

2-(*p*-*n*-Butylanilino)adenosine (4). To a solution of **3** (8.60 g, 15.9 mmol), tetraethylammonium chloride (8.52 g) and *N*,*N*-dimethylaniline (2.76 ml) in acetonitrile (120 ml) was added phosphoryl chloride (11.5 ml), and the mixture was heated for 1 h at 80 °C. After cooling, $CHCl_3$ (200 ml) was added to the mixture, which was then added dropwise into 40% (w/v) aqueous potassium carbonate (130 ml) with stirring. The separated organic phase was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was dissolved in methanolic ammonia (200 ml), and the mixture was heated for 5 h at 120 °C. The solvent was evaporated and the residual material was crystallized from ether/methanol (9:1, v/v) to afford **4** as yellowish fibers (4.80 g, 72.9%): mp 183—185 °C. UV (H_2O) λ_{max} 277, 243, and (0.01 N HCl) λ_{max} 272 nm. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (t, 3 H, CH_3 of butyl), 1.1-1.8 (m, 4 H, CH_2 x 2 of butyl), 2.59 (t, 2 H, Ar-CH_2), 3.63 (br s, 2 H, H-5'), 3.8-4.0 (m, 1 H, H-4'), 4.13 (br s, 1 H, H-3'), 4.48 (br s, 1 H, H-2'), 5.83 (d, 1 H, H-1'), 6.85 (s, 2 H, NH_2-6), 7.03 and 7.70 (d x 2, 2 H x 2, aromatic), 8.06 (s, 1 H, H-8), 8.71 (s, 1 H, NH-2). *Anal.* Calcd. for $C_{20}H_{26}N_6O_4$: C, 57.96; H, 6.32; N, 20.28. Found: C, 57.84; H, 6.34; N, 20.16.

2-(*p*-*n*-Butylanilino)-9-[3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]adenine (5). To a solution of **4** (1.89 g, 4.57 mmol) and imidazole (1.34 g) in *N*,*N*-dimethylformamide (DMF) (20 ml) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (TIPDSCl) (1.75 ml, 48 mmol). After stirring for 2.5 h at room temperature, the reaction mixture was slowly poured into ice-water with vigorous

stirring. The resulting precipitates were collected by filtration and washed well with water. The material was dissolved in $CHCl_3$ and the separated organic phase was dried over $MgCl_2$. After evaporation of the solvent, the residue was purified by column chromatography on silica gel (80 g) with CH_2Cl_2/C_2H_5OH (19:1) to give **5** (2.76 g, 92.1%) as a colorless foam. UV (MeOH) λ_{max} 278 and 248 nm. ^{1}H-NMR ($CDCl_3$) δ: 0.8-1.1 (m, isopropyl x 4 and CH_3 of butyl), 1.2-1.8 (m, 4 H, CH_2 x 2 of butyl), 2.59 (t, 2 H, Ar-CH_2), 4.09 (br s, 3 H, H-5' and 4'), 4.5-4.8 (m, 2 H, H-2' and 3'), 5.47 (s, 2 H, NH_2-6), 5.93 (d, 1 H, H-1'), 6.83 (br s, 1 H, NH-2), 7.10 and 7.53 (d x 2, 2 H x 2, aromatic), 7.74 (s, 1 H, H-8).

2-(*p*-*n*-Butylanilino)-9-[2-*O*-trifluoromethanesulfonyl-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]adenine (6). To a solution of **5** (1.76 g, 2.7 mmol), 4-dimethylaminopyridine (DMAP) (0.49 g) and triethylamine (0.56 ml) in pyridine (20 ml) was added trifluoromethanesulfonyl chloride (0.35 ml), and the mixture was stirred for 3.5 h at room temperature. The mixture was slowly poured into ice-water (150 ml) with stirring. After addition of $CHCl_3$ (100 ml) and stirring the mixture, the organic phase was separated and dried over $MgSO_4$. The solvent was evaporated and the residual syrup was purified by column chromatography on silica gel (50 g) with $CHCl_3$/ethyl acetate (4:1) to give (**6**) (1.68 g, 79%) as a pale yellowish syrup. This material was used next step without further purification.

2-(*p*-*n*-Butylanilino)-9-[2-deoxy-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl]adenine (7b). A mixture of **6** (2.55 g, 3.9 mmol) and lithium bromide (0.51 g) in hexamethylphosphoric triamide (HMPA) (20 ml) was stirred for 2.5 h at room temperature. The mixture was slowly poured into ice-water (500 ml) with vigorous stirring. The resulting precipitates were collected by filtration and washed well with water. The material was dissolved in $CHCl_3$ and the separated organic phase was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography on silica gel (50 g) with $CHCl_3$/ethyl acetate (4:1) to give 2'-bromide **7a** (1.54 g, 54.9%) as a pale yellowish syrup. To a solution of this material in toluene (20 ml) was added catalytic amount of α,α'-azobis(isobutyronitrile) (AIBN) and tri-*n*-butyltin hydride (4.5 ml). After stirring for 3 h

at 90 °C, the mixture was applied to a column of silica gel (25 g) and the column was washed with *n*-hexane (300 ml). Elution was performed with $CHCl_3$/ethyl acetate (4:1), the fractions containing **7b** were combined, and the solvent was removed under reduced pressure to give **7b** (0.82 g, 59.8%) as a solid. ^{1}H-NMR ($CDCl_3$) δ: 0.8-1.15 (m, isopropyl x 4 and CH_3 of butyl), 1.2-1.8 (m, CH_2 x 2 of butyl), 2.4-2.8 (m, 4 H, H-2' and Ar-CH_2), 3.7-4.1 (m, 3 H, H-5' and 4'), 4.77 (dd, 1 H, H-3'), 5.37 (br s, 2 H, NH_2-6), 6.23 (d, 1 H, H-1'), 6.83 (br s, 1 H, NH-2), 7.10 and 7.53 (d x 2, 2 H x 2, aromatic), 7.77 (s, 1 H, H-8). *Anal.* Calcd. for $C_{32}H_{52}N_6O_4Si_2$•0.25 H_2O: C, 59.55; H, 8.20; N, 13.02. Found: C, 59.41; H, 8.17; N, 12.91.

2-(*p*-*n*-Butylanilino)-2'-deoxyadenosine (8a). To a solution of **7b** (0.50 g, 0.78 mmol) in tetrahydrofuran (THF) (15 ml) was added 1 M THF solution of tetrabutylammonium fluoride (1.7 ml). The mixture was stirred for 2 h at room temperature. The mixture was condensed under reduced pressure. The resulting precipitates were collected by filtration and purified by column chromatography on silica gel (25 g) with $CHCl_3$/methanol (14:1). After evaporation of the solvent, the residue was crystallized from methanol to give **8a** as pale yellowish fibers (0.29 g, 92%): mp 168—170 °C (lit. 167—169 °C)[4]. HR-MS *m/z*: 398.2066 (M^+). Calcd. for $C_{20}H_{26}N_6O_3$: 398.2066.

9-[2-*O*-Acetyl-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-arabinofuranosyl]-2-(*p*-*n*-butylanilino)adenine (7c). A mixture of **6** (1.68 g, 2.13 mmol) and sodium acetate (0.2 g, 2.43 mmol) in HMPA (10 ml) was stirred for 4 h at room temperature. The reaction mixture was slowly poured into ice-water (200 ml) with vigorous stirring. The precipitates were collected by filtration and washed with water. After the material was dissolved in $CHCl_3$, the separated organic phase was washed with brine and dried over $MgSO_4$. After evaporation of the solvent, the residual material was purified by column chromatography on silica gel (50 g) with $CHCl_3$/ethyl acetate (4:1) to give **7c** (0.66 g, 44%) as a pale yellowish foam. ^{1}H-NMR ($CDCl_3$) δ: 0.9-1.2 (m, isopropyl x 4 and CH_3 of butyl), 1.3-1.7 (m, 4 H, CH_2 x 2 of butyl), 1.75 (s, 3 H, acetyl), 2.57 (t, 2 H, Ar-CH_2), 3.8-4.2 (m, 3 H, H-4' and 5'), 4.68 (dd, 1 H, H-3'), 5.57 (s, 2 H, NH_2-6), 5.64 (dd, 1 H, H-2'), 6.37 (d, 1 H, H-1'), 6.94 (s, 1 H, NH-2), 7.12 and 7.50 (d x

2, 2 H x 2, aromatic), 7.88 (s, 1 H, H-8). HR-MS *m/z*: 698.3643 (M^+). Calcd. for $C_{34}H_{54}N_6O_6Si_2$: 698.3643.

9-(β-D-Arabinofuranosyl)-2-(*p*-*n*-butylanilino)adenine (8b). To a solution of **7c** (0.60 g, 0.86 mmol) in THF (10 ml) was added 1 M THF solution of tetrabutylammonium fluoride (1.9 ml). The mixture was stirred for 4 h at room temperature. After evaporation of the solvent, the residue was dissolved in methanolic ammonia (40 ml) and the solution was allowed to stand for 14 h at room temperature. The mixture was evaporated to dryness, and the residue was purified by column chromatography on silica gel (40 g) with $CHCl_3/CH_3OH$ (19:1) to give **8b** (0.19 g, 53%) as a colorless foam. UV (H_2O) λ_{max} 278 nm (ε 21 000) and 243 nm (ε 19 700). ^{1}H-NMR (DMSO-d_6) δ: 0.93 (t, 3 H, CH_3 of butyl), 1.1-1.6 (m, 4 H, CH_2 x 2 of butyl), 3.4-4.2 (m, 5 H, H-3', 4' and 5'), 6.17 (d, 1 H, H-1'), 6.81 (s, 2 H, NH_2-6), 7.02 and 7.70 (d x 2, 2 H x 2, aromatic), 7.90 (s, 1 H, H-8), 8.69 (s, 1 H, NH-2). HR-MS *m/z*: 414.2018 (M^+). Calcd. for $C_{34}H_{54}N_6O_6Si_2$: 414.2015.

9-(2-Azido-2-deoxy-β-D-arabinofuranosyl)-2-(*p*-*n*-butylanilino)adenine (8c). A mixture of **6** (0.95 g, 1.21 mmol) and lithium azide (0.08 g) in HMPA (10 ml) was stirred for 2.5 h at room temperature. The reaction mixture was slowly poured into ice-water (200 ml) with vigorous stirring. The resulting precipitates were collected by filtration and washed with water. After the material was dissolved in $CHCl_3$, the separated organic phase was washed with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel (50 g) with $CHCl_3$/ethyl acetate (4:1) to give a pale yellowish foam (0.48 g, 58%). To a solution of this material in THF (10 ml) was added 1 M THF solution of tetrabutylammonium fluoride (1.54 ml), and the mixture was stirred for 14 h at room temperature. After evaporation of the solvent, the residue was purified by column chromatography on silica gel (40 g) with $CHCl_3/CH_3OH$ (19:1) to give **8c** as pale yellowish fibers (0.28 g, 91%, crystallized from CH_3OH): mp 221—224 °C. IR (KBr); 2100 cm^{-1} (azido). UV (H_2O) λ_{max} 278 and 243 nm. ^{1}H-NMR (DMSO-d_6) δ: 0.90 (t, 3 H, CH_3 of butyl), 1.1-1.7 (m, 4 H, CH_2 x 2 of butyl), 3.6-3.9 (m, 3 H, H-4' and 5'), 4.3-4.6 (m, 2 H, H-2' and 3'), 6.34 (d, 1 H, H-1'), 6.85 (s, 2 H, NH_2-6), 7.02 and 7.70 (d x

2, 2 H x 2, aromatic), 8.00 (s, 1 H, H-8), 8.69 (s, 1 H, NH-2). *Anal.* Calcd. for $C_{20}H_{25}N_9O_3$ 1/3CH_3OH: C, 54.26; H, 5.89; N, 28.01. Found: C, 54.29; H, 5.88; N, 28.07.

BuAaraATP (9b) and AzBuAaraATP (9c). To a solution of BuAaraA (**8b**) (50 mg, 0.12 mmol) in triethylphosphate (1 ml) was mixed with phosphoryl chloride (0.088 ml, 0.96 mmol) at –10 °C and the mixture was stirred for 24 h at 4 °C. After the mixture was neutralized by addition of saturated aqueous $NaHCO_3$ (10 ml), $CHCl_3$ (10 ml) was added to the mixture. The aqueous phase was separated and diluted with water to 80 ml. The solution was applied to a column of DEAE-cellulose (2.5 x 10 cm, bicarbonate form), which was eluted with a liner gradient (1,400 ml) of 0 to 0.7 M triethylammonium bicarbonate (TEAB) (pH 7.8). The main peak of UV absorption was observed at 0.5 M TEAB and was corresponding to BuAaraA 5'-monophosphate (BuAaraAMP). The fractions were combined and evaporated to dryness to give BuAaraAMP (1288 OD_{278} units, 0.61 mmol, 51%). To a solution of BuAaraAMP (630 OD_{278} units, 0.03 mmol) in DMF (1 ml) was added 1,1'-carbonyldiimidazole (24 mg), and the solution was stirred at room temperature for 5 h. After treatment with methanol (5 μl) for 0.5 h at room temperature, the reaction mixture was mixed with DMF solution of 0.6 M tri-*n*-butylammonium pyrophosphate. The mixture was stirred at room temperature for 15 h. After evaporation of the solvent, the residue was dissolved in 20% acetonitrile. The solution was applied to a column of DEAE-cellulose (1.5 x 17 cm, bicarbonate form), which was eluted with a liner gradient (500 ml) of 0 to 0.75 M TEAB (pH 7.8) containing 20% acetonitrile. The fractions containing 5'-triphosphate were combined and the solvent was removed under reduced pressure to give crude **9b** (90 OD_{278} units, 14%). A portion of the material was further purified by HPLC (Method A).

In a similar fashion, AzBuAaraATP (**9c**) was synthesized from AzBuAaraA (**8c**).

When analyzed by HPLC (Method A), retention times of **9b** and **9c** were 5.7 and 10.3 min, respectively, and the purities of both compounds assessed by measuring UV absorption at 280 nm were confirmed to be greater than 95%.

Determination of Antitumor Activity. Mouse P388 cells were cultured in RPMI 1640 (Nissui Pharmaceutical) medium containing 10% fetal calf serum and 50 μM 2-mercaptoethanol. An aliquot of 200 μl of cell suspension (1 x 10^4 cells/ml) was seeded

into each well of a 96-well plate. After culture for 1 day at 37 °C under a 5% CO_2 atmosphere, the cells were treated with various concentrations of drugs in triplicate. After further culture for 2 days, cell growth was determined by the MTT method.[24]

Assays of Pol α, β and ε Activities. For the assay of pol α and ε activities a reaction mixture (25 μl) comprised 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 4 mM $MgCl_2$, 80 μg/ml calf thymus activated DNA, 50 μM dTTP, dCTP and dGTP, 50 μM [^{3}H]dATP (220 cpm/pmol) and 1 μl enzyme preparation (0.08 unit). For the assay of pol β, 50 mM Tris-HCl (pH 8.8) in place of the buffer (pH 7.5) was used and 100 mM KCl was added. Incubation was carried out for 10 or 20 min at 37 °C, and the radioactivity in the polynucleotides was measured[25]. One unit of pol was defined as the amount of enzyme that incorporated 1 nmol dTMP into polymer per 60 min at 37 °C.

Analyses of the effects of inhibitors were performed as follows. Activity without inhibitor was taken as 100%, and the remaining activities in the presence of various concentrations of inhibitors were measured. The mode of inhibition was analyzed by changing the concentrations of both inhibitor and [^{3}H]dATP.

Primer Extension Assay. The primer was labeled by incubating equal quantities of the primer (as 5'-OH) and [γ-^{32}P]ATP with T4 polynucleotide kinase under the conditions suggested by Nippon Gene. A 5' ^{32}P-labeled 14-mer primer was annealed to a 25-mer template (Fig. 3) by heating at 65°C for 5 min, followed by gradual cooling to room temperature. The reaction mixture (10 μl) comprised 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 4 mM $MgCl_2$, 1 μM 5'-[^{32}P]primer-template, various concentrations of dNTPs and inhibitors, and 2 μl of enzyme preparation. After incubation at 37°C for 20 min, the reactions were quenched by adding 1 volume of gel loading solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). Samples were denatured at 95°C for 5 min and loaded on 20% polyacrylamide-7 M urea DNA sequencing gels. Electrophoresis was carried out at 40 W for 3-4 h. After drying the gel, the radioactive products were detected by autoradiography.

Purification of Pol ε and PCNA from Immature Cherry Salmon Testis. DNA polymerase ε was purified from a crude extract prepared from immature testis of cherry

salmon by means of sequential column chromatography on phosphocellulose (P11, Whatman), DEAE-cellulose (DE52, Whatman), phenyl-Sepharose (Pharmacia Biotech) and hydroxyapatite (HTP GEL, Bio-Rad). PCNA was also purified from the same starting material according to the method described by Prelich *et al.*[26] The final preparation of pol ε had a specific enzyme activity of 1,500 units/mg protein with activated DNA as a template-primer, having been purified 1,500-fold from the crude extract, and contained polypeptides of *Mr* 110,000 and small amount of *Mr* 50,000. The pol obtained was identified as pol ε because of its inhibition by aphidicolin (IC_{90} = 10 μg/ml). However, it was distinguishable from pol α by the presence of 3'–5' exonuclease activity and lack of primase activity. This polymerase activity utilized poly(dA)/oligo(dT) as well as activated DNA and was independent of PCNA.

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